

Systematic Studies of *Polystichum* (Dryopteridaceae) in Japan (I): *P. fibrillosopaleaceum* var. *marginale* is a Diploid Hybrid between *P. fibrillosopaleaceum* and *P. igaense*

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ABSTRACT.—Morphological, cytological, and isozyme studies of three plants of *Polystichum fibrillosopaleaceum* var. *marginale* (Dryopteridaceae) from Gotenba, at the foot of Mt. Fuji, Shizuoka Prefecture, Japan, and comparative observations of their lamina, scales, sori, spores, and perispore ornamentation, revealed them to be intermediate between *P. fibrillosopaleaceum* and *P. igaense*. Cytologically, the meiotic chromosome number of $n=c. 38II + 6I (2x)$ and the malformed spores of the three plants are characteristic of hybrid sterility. The electrophoretic polymorphisms of four isozymes (Pgi, Pgm-1, Pgm-2, and Skdh) were examined in the three plants and in 22 individuals of the putative parental species, *P. fibrillosopaleaceum* (11) and *P. igaense* (11). The heterozygous genetic patterns (Pgi-ab, Pgm-1ab, Pgm-2ab, and Skdh-ab) fixed in the putative hybrids indicate that they have a combination of the Pgi-b, Pgm-1b, Pgm-2a, and Skdh-a alleles of *P. igaense* and Pgi-a, Pgm-1a, Pgm-2b, and Skdh-b alleles of *P. fibrillosopaleaceum*. The morphological, electrophoretic features and chromosome numbers confirm the three plants to be diploid hybrids of *P. fibrillosopaleaceum* and *P. igaense*. We therefore conclude that *P. fibrillosopaleaceum* var. *marginale* is in fact a diploid hybrid and thus should not be given the variety status.

KEY WORDS.—*Polystichum*, hybrid, diploid, variety, electrophoretic polymorphisms

Polystichum (Dryopteridaceae) is a genus estimated to contain about 200 to more than 300 species, occurring in mountainous, warm temperature areas, especially in eastern to southeastern Asia (Kramer and Green, 1990; Iwatsuki, 1992; Kung *et al.*, 2001, Little and Barrington 2003). Hybridization and polyploidization have played important roles in species diversification and reticulation in *Polystichum*, reflected in the high frequency of tetraploid species and hybrids (Daigobo, 1972; Vida and Reichstein, 1975; Wagner, 1973; Wagner, 1979; Barrington, 1985, 1986, 1990; Soltis and Soltis, 1987). Systematic studies have been undertaken to resolve the evolutionary and taxonomic questions regarding *Polystichum* in Europe and America during the

past half century (Manton, 1950; Wagner, 1973; Wagner, 1979; Barrington, 1986, 1990; Little and Barrington, 2003), and in Asia recently (Zhang and Kung, 1996; Lu *et al.*, 2007; Li *et al.*, 2008).

Based on morphological characters, 32 species and about 40 interspecific hybrids involving 13 putative parental species are recognized in Japan (Iwatsuki, 1992). It is generally difficult to confirm the taxonomy of closely related species or discriminate between them using only a single analytical method because the distribution of character states can overlap. It is therefore necessary to combine morphological, cytological, and electrophoretic analyses to determine the evolutionary relationships among the species in Japan.

Among the 13 Japanese putative parental species classified in sect. *Metapolystichum* (Daigobo 1972), six are diploid species (Takamiya 1996), for which systematic studies are few. It is important to clarify the identity of the diploid species before we can understand the origins of the hybrids and the polyploid species, and to resolve the history of speciation, hybridization and polyploidization in Japanese *Polystichum*. This study focused on the diploid species *P. igaense* Tagawa and *P. fibrillosopaleaceum* (Kodama) Tagawa, which are believed to be involved in the origin of more than 10 hybrids (Iwatsuki, 1992). *Polystichum igaense*, endemic to Japan, occurs in mountain forests on Honshu, Shikoku, and Kyushu. *Polystichum fibrillosopaleaceum*, a well-known Japanese endemic species, is widely distributed on the Pacific Ocean side of Japan from Honshu and Shikoku to Kyushu in forests around lowland villages, overlapping locally with *P. igaense*.

Plants from Shizuoka Prefecture, Honshu Island, Japan have been segregated as *Polystichum fibrillosopaleaceum* var. *marginale* Seriz. With only a few individuals found in the wild, it is endangered, classified as grade IA (CR) in the Red Data Book of the Japanese Environmental Agency (2007). *Polystichum fibrillosopaleaceum* var. *marginale* has morphological features similar to those of *P. fibrillosopaleaceum*, such as nearly entire, twisted scales, deltoid outline of pinnules, and conspicuous fibrillose scales on the undersurface of the pinnules, but other characters, such as shorter stipes, untwisted pinna and rachis scales, and marginal sori, are similar to the features of *P. igaense* (Serizawa, 1971). Serizawa (1971) considered temporarily that it might be a hybrid between *P. igaense* and *P. fibrillosopaleaceum*. The meiotic chromosome number of *P. fibrillosopaleaceum* var. *marginale* is reported to be $n = 41$ II (2x), the same as that of *P. fibrillosopaleaceum* (Daigobo, 1973; Shimura and Ooishi, 1980). Those early studies support the proposition that *P. fibrillosopaleaceum* var. *marginale* is probably a variety of *P. fibrillosopaleaceum*, although irregular sterile spores are sometimes produced (Shimura, 1975), and Nakaike (1992) elevated it to species as *P. shizuokaense* Nakaike. Lack of detailed studies of *P. fibrillosopaleaceum* var. *marginale*, however, has hindered further classification of this taxon.

In the present study, three individuals were collected in the area where *P. fibrillosopaleaceum* and *P. igaense* occur in sympatry; they were tentatively identified as *P. fibrillosopaleaceum* var. *marginale* on the basis of their morphological features. To understand the nature and evolutionary origin of *P.*

TABLE 1. Materials used in morphology, cytology and electrophoresis.

Taxa	Locality & voucher no.	Chromosome no. (n, 2n)	Electro. examined samples
<i>P. fibrillosopaleaceum</i> (2x)	Asahi, Yokohama, Kanagawa Pref. (40215, 06100105)	2n=c.82	2
	Midori, Yokohama, Kanagawa Pref. (40039, 40050)		
	Fujisawa, Kanagawa Pref. (40219, 40221, 40223)		3
	Gotenba, Shizuoka Pref. (40013, 40021, 40036; 40230, 40242, 40253)		3
	Kannan City, Shizuoka Pref. (05060727, 05060729)		2
	Misato, Gunma, Gunma Pref. (40513, 40514, 40515)		1
Hybrid (2x) (<i>P.</i> <i>fibrillosopaleaceum</i> var. <i>marginale</i>)	Gotenba, Shizuoka Pref. (40238) (40018, 40020)	n=c.41	3
<i>P. igaense</i> (2x)	Gotenba, Shizuoka, Pref. (40006, 40009, 40022, 40033, 40038; 40227, 40228, 40234, 40245, 06100302)	2n=82	5
	Susono, Shizuoka Pref. (40260, 40261, 05060519, 05060520, 05060531, 06100344)		6

fibrillosopaleaceum var. *marginale*, morphological, cytological and electrophoretic analyses were carried out to compare these three plants with *P. fibrillosopaleaceum* and *P. igaense*.

MATERIALS AND METHODS

Thirty herbarium specimens of *Polystichum* from Japan, deposited in the herbaria of Botanical Garden, University of Tokyo (TI), the Department of Botany, Kyoto University (KYO), and the Japanese National Museum of Nature and Science (TNS), were used for morphological observations. Three samples collected from Gotenba, Shizuoka Prefecture, which had previously been presumed to be *P. fibrillosopaleaceum* var. *marginale*, were examined and compared morphologically with *P. fibrillosopaleaceum* and *P. igaense*. The habitat of the three taxa overlapped in a very narrow area (about 5 × 5 m²) in an evergreen forest (cedar) in Gotenba.

For cytological and electrophoretic analyses, the three samples of *P. fibrillosopaleaceum* var. *marginale* from Gotenba, Shizuoka Prefecture, 11 samples of *P. fibrillosopaleaceum* and 11 samples of *P. igaense* were collected in Shizuoka and Kanagawa Prefectures (Table 1). The voucher specimens for these analyses are deposited in the herbarium, Department of Biological Science, Faculty of Life and Environmental Science, Shimane University, Japan.

Spores were obtained from fertile leaves. Spore form and spore number per sporangium (s/s) were observed under a light microscope. The spores obtained from the type specimen of *P. fibrillosopaleaceum* var. *marginale* (Serizawa 6180 = TNS VS-573649) and the three samples collected from Godenba (40018, 40020, and 40238) were compared. To observe perispore ornamentation, dried spores were coated with gold under vacuum with no pretreatment (E-102, Pt-Pd, 3 minutes, 100 Å) and observed by scanning electron microscopy (SEM; S-800 Hitachi). To examine spore germination, spores were gathered from mature sporangia at the stage just before opening to avoid contamination, sown on standard 1/10 MS medium, and cultured at 20–25°C under uninterrupted fluorescent light of about 800–1000 lux.

For cytological analysis, root tips were pretreated with 0.002 M 8-hydroxyquinoline solution for 5 hrs at 20 °C, fixed in an acetic acid–alcohol (1:3) solution for more than 15 min, macerated in a mixed solution of 1 N hydrochloric acid (HCl): 45% acetic acid (3:1) for 2.5 min at 58 °C, and stained with 2% aceto–orcein. The root tissue (about 1–2 mm of root tip) was then cut and squashed for somatic observation (Lin *et al.*, 1990). Fresh pinnae of fertile leaves with sori were fixed in an acetic acid–alcohol (1:3) solution (more than 15 minutes) and then stored at 4–10°C for meiotic observation.

Flow cytometric analysis was used to confirm the ploidy level of those samples for which cytological materials could not be obtained. Approximately 1cm² fresh leaf tissue was chopped with a razor, and subsequent methods followed Ebihara *et al.* (2005).

For electrophoretic analysis, fresh leaf material (100 mg) from each sample was ground in 0.5 ml of Tris-HCL extraction buffer pH 7.5 (Soltis *et al.*, 1983). The slurry was centrifuged and the supernatant was subjected to electrophoresis on 10% polyacrylamide gels. Five isozyme systems were examined: aspartate aminotransferase (Aat), leucine aminopeptidase (Lap), phosphoglucosomutase (Pgm), phosphoglucose isomerase (Pgi), and shikimate dehydrogenase (Skdh). All isozymes migrated anodally. When the isozymes were encoded by more than one locus, the most anodally migrating isozyme was designated 1, the next 2, and so on.

RESULTS

Morphology.—The three samples from Godenba were observed morphologically and identified to be *P. fibrillosopaleaceum* var. *marginale* based on the characters such as nearly entire and twisted scales, and submarginal sori as described by Serizawa (1971). The average size and shape of the leaves of the samples (40018, 40020, and 40238) showed morphological features intermediate between those of *P. fibrillosopaleaceum* and *P. igaense* (Figs 1–2; Table 2). The stipes of the three plants were 15.4 (\pm 5.1) cm long with dense scales. The lanceolate lamina was 54.8 (\pm 9.7) cm long and 16.6 (\pm 2.1) cm wide, longer than that of both species. The scales on the lower portion of the stipes were slightly tortuous, and the margins of the scales were more or less denticulate with sparse projections more similar to those of *P. igaense*, which

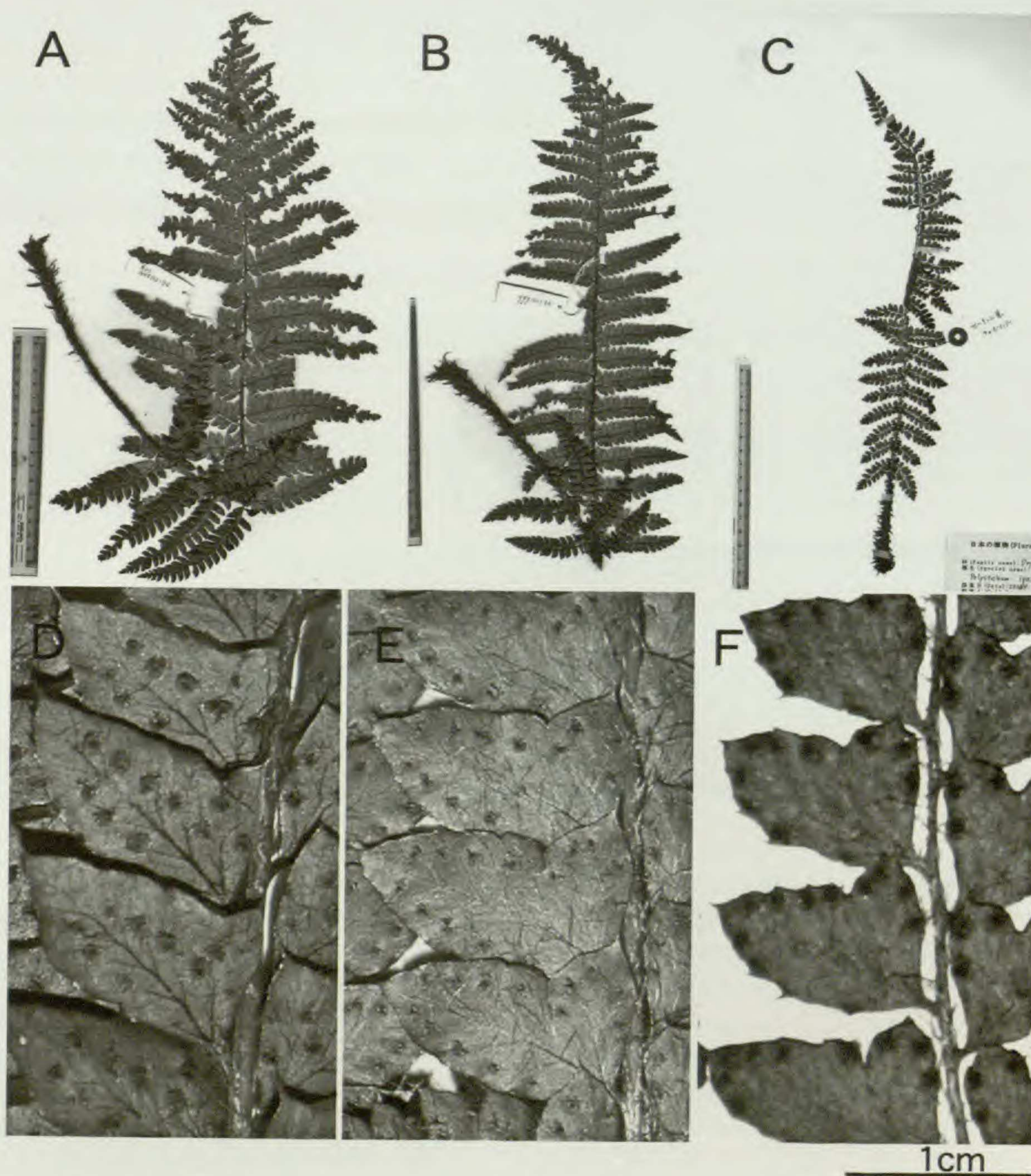


FIG. 1. Leaf photographs. A, D: *P. fibrillosopaleaceum*; B, E: *P. fibrillosopaleaceum* var. *marginale*; C, F: *P. igaense*.

has scales with projection margins, slightly tortuous when dried (the scales of *P. fibrillosopaleaceum* have entire margins and are strongly twisted). The sori were marginal or submarginal on the pinnules, like those of *P. igaense*.

The spores of *P. fibrillosopaleaceum* and *P. igaense* are normally 64 s/s and monolet, with distinct features in the perispore. Those of *P. fibrillosopaleaceum* are reticulate echinate, whereas those of *P. igaense* are fenestrate cristate (Fig. 3). The spores of the three samples were highly irregular in size (30–60 μm) and number (32 s/s, c. 48 s/s, 64 s/s, c. 112 s/s), and were malformed,

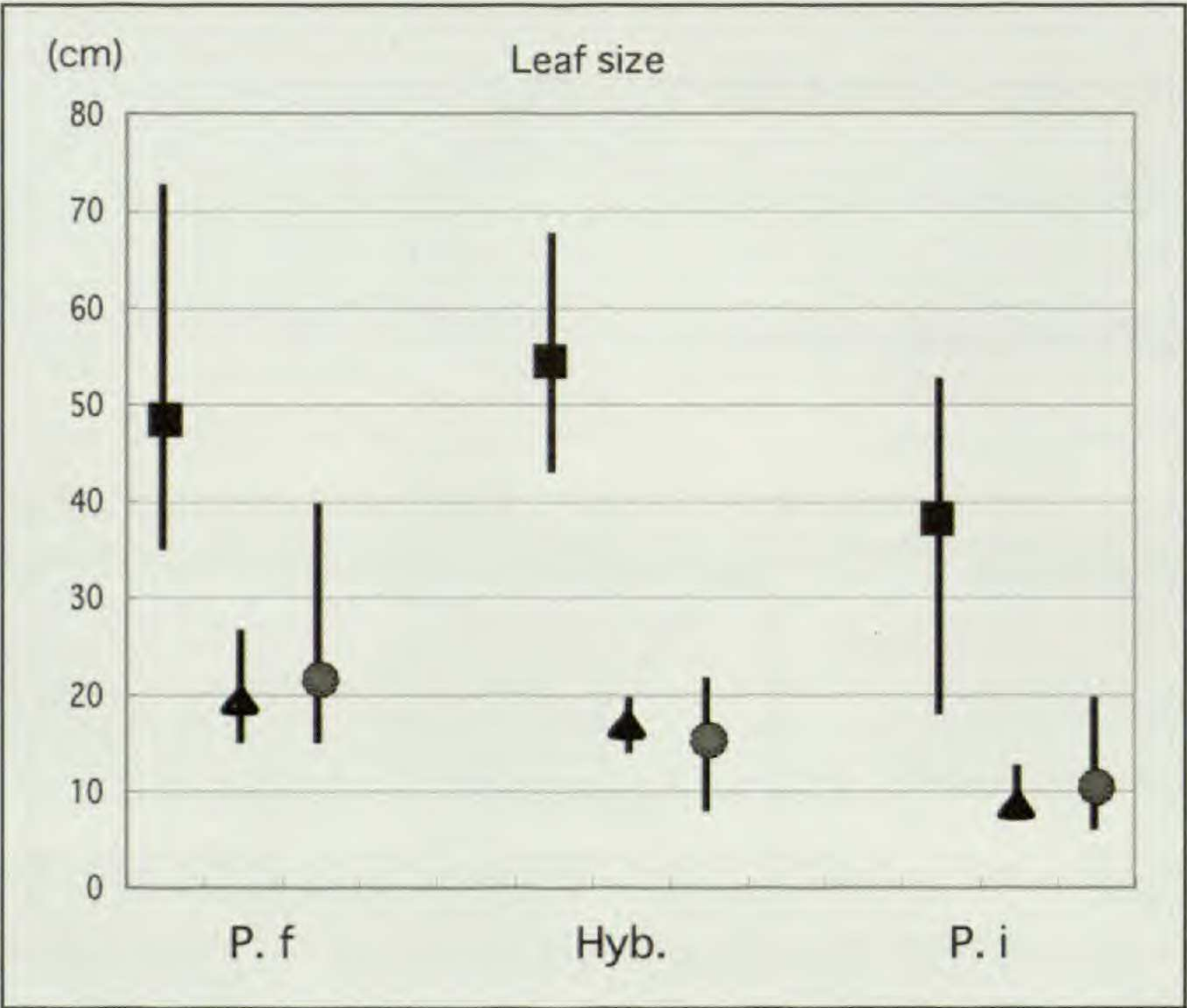


FIG. 2. Leaf size data. Squares show frond lengths; triangles show the widths of fronds; circles show the lengths of stipes.

and of three different types: reticulate echinate (like *P. fibrillosopaleaceum*), fenestrate cristate (like *P. igaense*), and cristate with coalescent echinate. These types were observed either in single sporangia or in different sporangia of a single plant (Fig. 3B, D). Three herbarium specimens of *P. fibrillosopaleaceum* var. *marginale* including a holotype specimen (Serizawa 6180 = TNS VS-573649) were studied for comparison. The spores obtained from the holotype specimen (Fig. 4) and specimens 40018, 40020, and 40238 were also irregular, and with variable perispores.

TABLE 2. Morphological characters in the putative hybrid and in its putative parent species *P. fibrillosopaleaceum* and *P. igaense*. **P.f:** *P. fibrillosopaleaceum*; **Hyb.:** the putative hybrid; **P.i:** *P. igaense*.

Taxa	P.f	Hyb.	P.i
Characters			
Leaf shape (cm)			
Laminae length	48.4±12.5	54.8±9.7	37.3±9.1
Laminae width	19.1±3.4	16.6±2.1	8.7±1.4
Stipes	21.7±7.0	15.4±5.1	10.5±2.9
Scales (length, cm)	tortuous, entire (1.45±0.29)	a little tortuous, sparsely projects (1.37±0.40)	a little tortuous when dry, pro- jects (0.87±0.16)
Sori	medial	marginal or submarginal	marginal
Spore	reticulate echinate	irregular	cristate

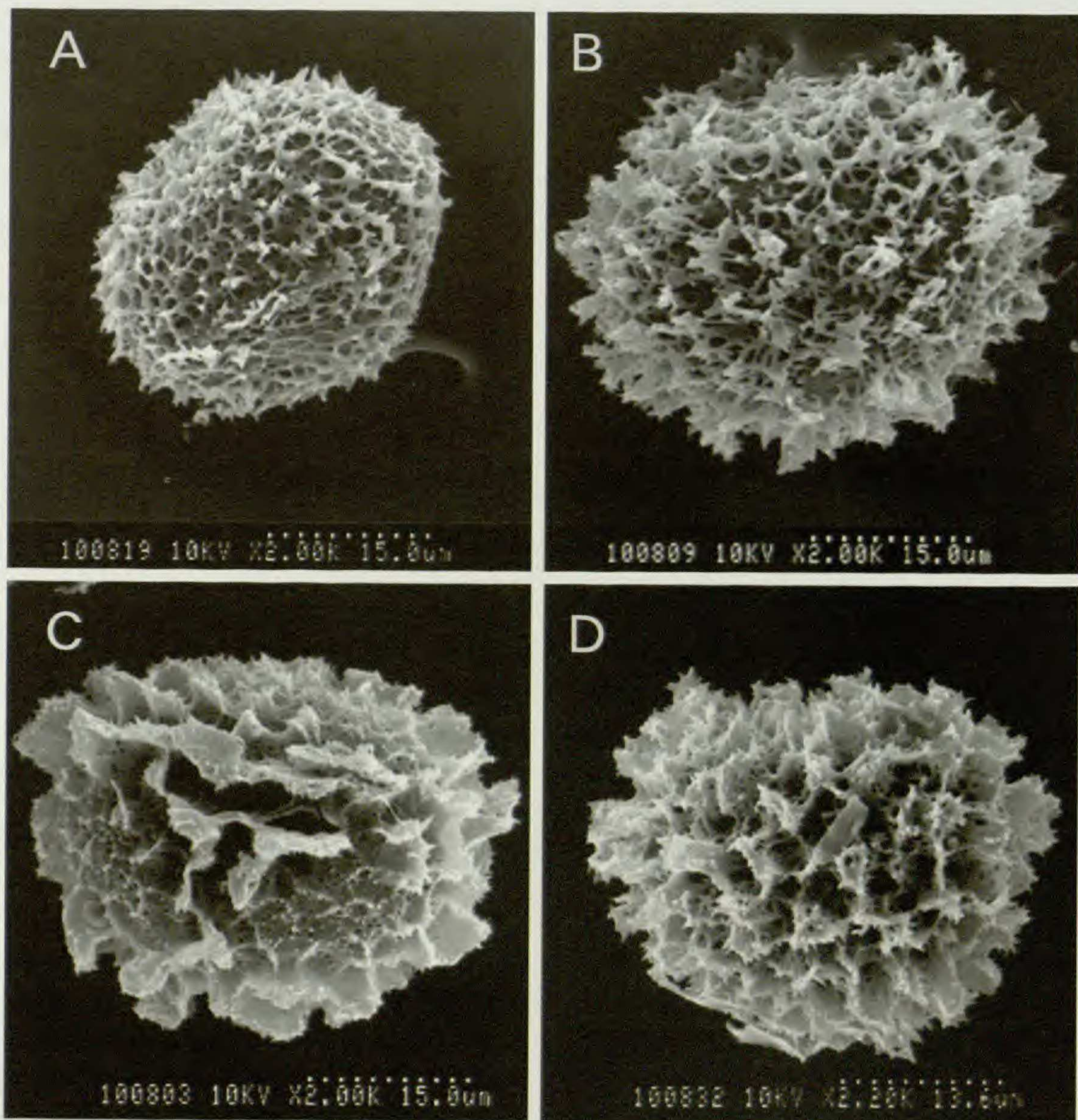


FIG. 3. SEM images of spore morphology. A: *P. fibrillosopaleaceum*; C: *P. igaense*; B, D: *P. fibrillosopaleaceum* var. *marginale*.

The spore viability of an individual sample (40238) was tested. Only about 1% of the spores germinated and grew to prothalli within 3–4 months of sowing, whereas about 75% of the control samples (viable spores) from a plant of *P. fibrillosopaleaceum* germinated in 1 month. The prothalli of 40238 were irregular or cordate in shape, and there were several archegonia (which soon withered) in the cushion zone, but no antheridia or young sporophytes were observed before the prothalli died.

Cytological evidence and polyploidy.—The chromosome numbers, meiotic behavior and polyploidy of *P. fibrillosopaleaceum*, *P. igaense*, and the three samples of *P. fibrillosopaleaceum* var. *marginale* were examined (Table 1). Both *P. fibrillosopaleaceum* and *P. igaense* were diploid with $2n = c. 82$ chromosomes (Fig. 5A–D), consistent with previous studies (Mitui, 1965, 1966, 1968; Kurita, 1966; Daigobo, 1973). Because the suitable cytological

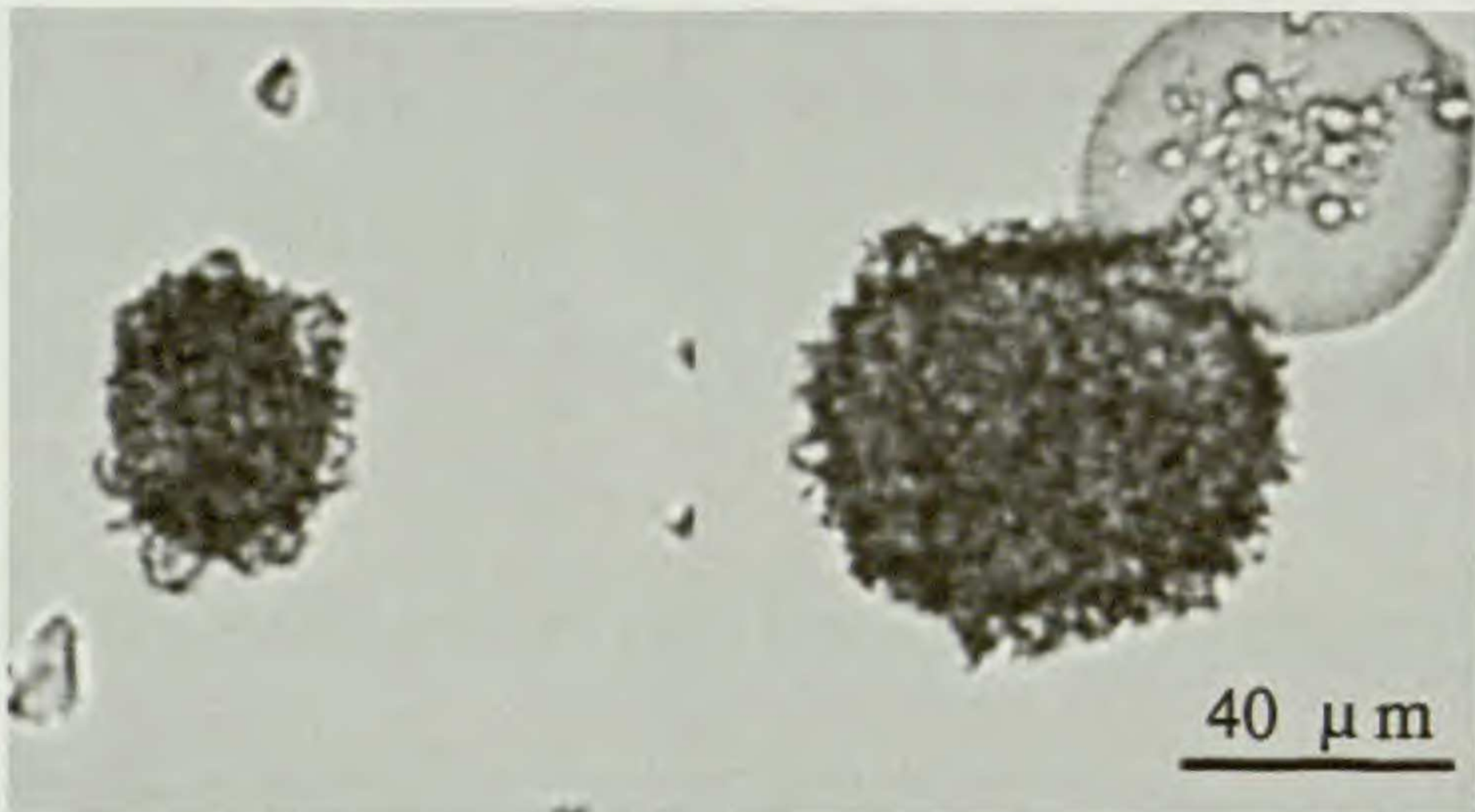


FIG. 4. Spores of *P. fibrillosopaleaceum* var. *marginale* (holotype specimen 6180).

materials could not be obtained in field for two of the three *P. fibrillosopaleaceum* var. *marginale* samples (40018 and 40020), the both samples were examined by flow-cytometric analysis, and were shown to be diploid by flow-cytometric analysis. The meiotic behavior of the other sample (40238) was observed. Occasional irregular meiotic division, such as a chromosome bridge at anaphase II, and chromosomal fragments at metaphase I and telophase II were observed (Fig. 6A–C). However, a few normal-looking meiotic mother cells, with approximately 41 II bivalent chromosomes, were observed in the same plant (Fig. 5E–F), and it was presumed that such sporocytes could produce normal tetrads. Various sporangia (with 16 sporocytes) were observed in this individual, which ranged from almost normal (64 s/s) to extremely irregular (c. 48 s/s, 32 s/s, and c. 112 s/s). The variability of the sporangia suggests genetic instability during meiosis.

Isozyme analysis.—Seven loci were clearly interpretable: Aat, Lap, Pgi, Pgm-1, Pgm-2, Pgm-3, and Skdh. Aat, Lap, and Pgm-3 were monomorphic and subsequently omitted from the analysis. *Polystichum fibrillosopaleaceum* and *P. igaense* have different alleles at the loci encoding Pgi, Pgm-1, Pgm-2, and Skdh. None of the genetic markers present in all 11 samples of *P. igaense* (Pgi-b, Pgm-1b, Pgm-2a, and Skdh-a) were found in *P. fibrillosopaleaceum*. Conversely, the alleles Pgi-a, Pgm-1a, Pgm-2b, and Skdh-b were detected in all 11 individuals of *P. fibrillosopaleaceum* but absent in the samples of *P. igaense*. The three putative hybrids showed the same heterozygous 4-locus genotypes, Pgm-1ab, Pgm-2ab, Pgi-ab, and Skdh-ab, a combination of alleles from both *P. fibrillosopaleaceum* and *P. igaense* (Fig. 7).

DISCUSSION

In general, intermediate morphological characters and irregular, sterile spores suggest hybridity in ferns. All morphological characters of the three *P. fibrillosopaleaceum* var. *marginale* samples examined here showed a combi-

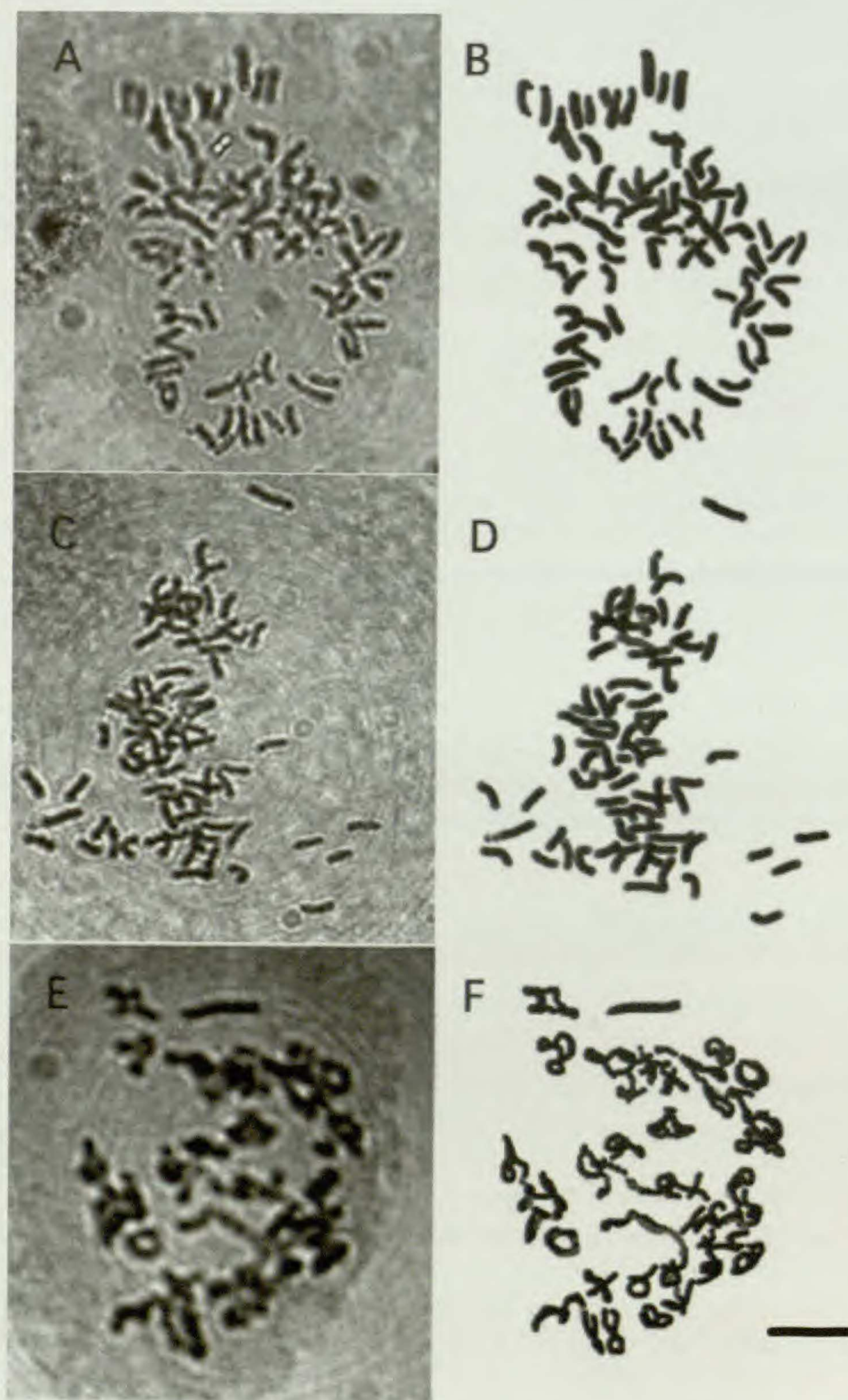


FIG. 5. Photographs of chromosomes. A, B: *P. fibrillosopaleaceum*, $2n = 82$ (2x); C, D: *P. igaense*, $2n = 82$ (2x); E, F: *P. fibrillosopaleaceum* var. *marginale*, $n = c. 41$ (2x). (bar = 10 μm).

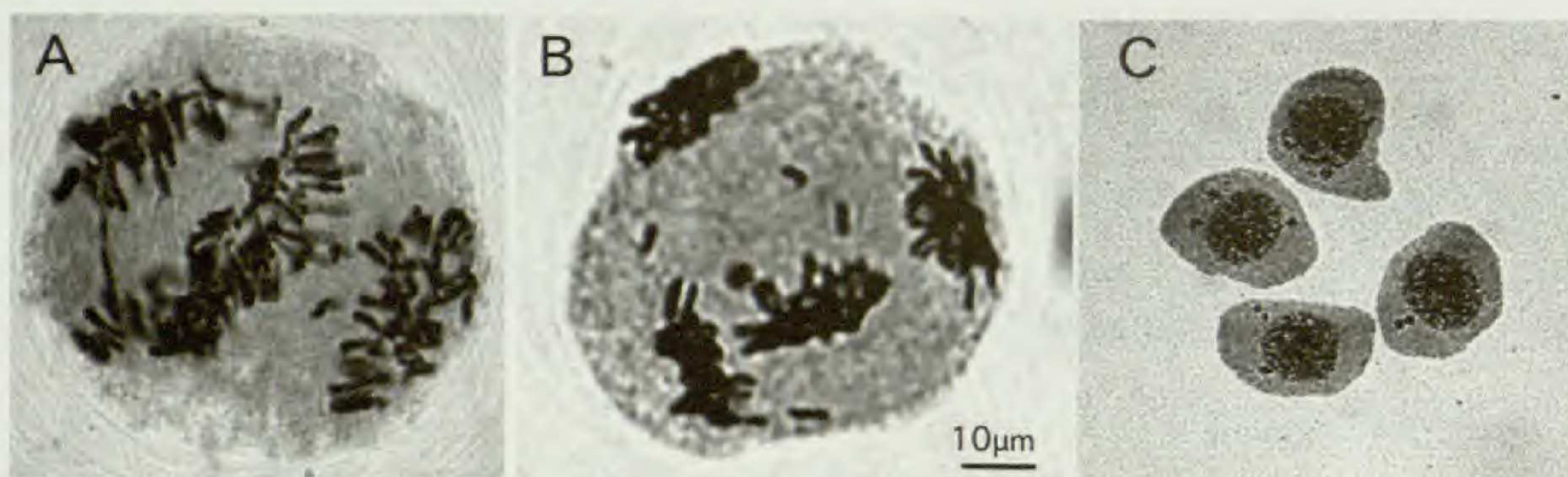


FIG. 6. Photographs of tetrads of *P. fibrillosopaleaceum* var. *marginale*. A: showing a bridge in spore mother cell at meiotic anaphase II; B: showing some fragments or univalent chromosomes in spore mother cell at meiotic telophase II; C: tetrad with chromosome fragments.

nation of the features of *P. fibrillosopaleaceum* and *P. igaense*, or similarity with the form of *P. fibrillosopaleaceum* or *P. igaense*. Chromosome numbers and flow-cytometric data showed both the putative hybrids and their putative parents to be diploid ($n=41$ and $2n=82$). The presence of one to several univalent chromosomes or fragments at metaphase I or telophase II and anaphase II suggested the lack of full homology in the pairing of homologous chromosomes. There appears to be an association between lack of chromosome pairing and irregular spores and reduced spore germination in these plants. The electrophoretic evidence demonstrates that the genomes of the putative hybrids are a combination of contributions from the diploid progenitors, *P. fibrillosopaleaceum* and *P. igaense*. All results obtained in this study support our hypothesis that the three samples of *P. fibrillosopaleaceum* var. *marginale* are diploid hybrids derived from the disparate diploid species *P. fibrillosopaleaceum* and *P. igaense*.

In the dataset for DNA barcoding of Japanese pteridophytes (Ebihara *et al.*, 2010), *P. fibrillosopaleaceum* var. *marginale* showed almost identical chloroplast *rbcL* sequences as *P. igaense*. This suggests *P. igaense* is probably the maternal parent species of *P. fibrillosopaleaceum* var. *marginale*. These data further support the idea that *P. fibrillosopaleaceum* var. *marginale* could be of hybrid origin.



FIG. 7. Photographs showing electrophoretic band patterns of Pgi, Skdh, and Pgm. (I: *P. igaense*; F: *P. fibrillosopaleaceum*; H: Putative hybrid (*P. fibrillosopaleaceum* var. *marginale*).

Although abnormal spores were observed on some specimens, Serizawa (1971) considered *P. fibrillosopaleaceum* var. *marginale* to be a variety of *P. fibrillosopaleaceum* rather than a hybrid, because Daigobo (personal communication) found that its meiosis was normal with a complement of 41 II chromosomes. Daigobo (1974) and Shimura (1975, 1980) also reported a normal complement of 41 II chromosomes, but Shimura (1980) noted irregular spores and a germination rate of only 2~7.5% in *P. fibrillosopaleaceum* var. *marginale*.

A similar phenomenon was also observed in the hybrid between *P. concinnum* and *P. speciosissimum*, which showed virtually complete chromosomal pairing in some cells, even though the progenitor species are strongly differentiated morphologically (Barrington, 1990). Barrington (1990) reported that in such situations, chromosome pairing may be under less stringent genetic control in *Polystichum* than in other fern groups, thus allowing the relatively high frequencies of homologous and homoeologous pairing as observed in *Polystichum* hybrids. Thus it is very difficult to morphologically distinguish the hybrids of closely allied species of *Polystichum*, because of their continuous morphological variation. *Polystichum fibrillosopaleaceum* is considered to be involved in several hybrids as a putative diploid parent (Iwatsuki, 1992) and as the ancestor of various polyploid species (Lin *et al.*, unpublished data). Therefore it can be concluded that *P. fibrillosopaleaceum* plays an important role in the hybridization, polyploidization, and reticulate evolution of Japanese *Polystichum*.

Taxnomic treatment:

Polystichum* x *shizuokaense Nakaike, pro sp.—*Polystichum shizuokaense* Nakaike, New Fl. Jap. Pterid. 842 (1992) —*Polystichum fibrillosopaleaceum* (Kodama) Tagawa var. *marginale* Seriz., J. Geobot. 19: 104 (1971).

ACKNOWLEDGMENTS

Drs. A. Ohshima, M. Takamiya, T. Suzuki, and H. Ishikawa, Mr. T. Oka and Ms. M. Nagamoto are thanked for their help during fieldwork, for their technical assistance and for helpful discussions. Thanks are also due to Dr. David E. Boufford for his useful comments and help with revising the manuscript. The curators of TI, KYO, TNS and HUH are acknowledged for their kind permission to examine specimens during this study, and the Department of Organismic and Evolutionary Biology, Harvard University, is acknowledged for offering S.-J. Lin (the senior author) a comfortable research environment during Lin's sabbatical period. This study was partly supported by the New Technology Development Foundation, Showa Seitoku Memorial Foundation, and the Science Project (project no. 19570087 for S.-J. Lin) of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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